Effect of Exposure Time, Cell Concentration and Culture Age on the Microbial Adhesion of *Staphylococcus Aureus and Saccharomyces Cerevisiae* on the Glass Surface

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ABSTRACT

This paper reveals the behaviour of the adhesion of *Staphylococcus aureus* (*S. aureus*) and *Saccharomyces cerevisiae* (*S. cerevisiae*) on the glass surface with respect to the exposure time, the cell concentration and culture age. Therefore, this study was carried out to investigate the mechanism of microbial adhesion based on the exposure time, cell concentration and culture age, so that further measures can be taken either to influence or to prevent the adhesion of microorganism. In order to investigate effect of exposure time on the adhesion process, the experiments were carried out for 24 hours and sampling was done at 4 h, 8 h, 12 h and 24 h of intervals. At each sampling time, glass slide was examined under the light microscope for determination of the numbers of cell attached per square area. Besides that, the absorbance and the colony forming unit (CFU) were also measured. Based on the result obtained, the absorbance for *S. cerevisiae* and *S. aureus* decreased with increasing exposure time with lowest OD reading was obtained at 24 hours, 0.911 and 0.827, respectively indicating a reduction by 8.9 % and 17.3 % from initial reading of 1.000. The OD reduction of cell suspension occurred because the bacteria were attached on glass surfaces and the degree of attachment increased with exposure time. This reading was supported by analysis of the colony forming unit (CFU) count, where the initial reading of CFU for *S. cerevisiae* was at 16 $x10^{11}$ CFU/ml and dropped to 10.3 $x10^{11}$ CFU/ml at the end of the experiment whereas for *S. aureus* the CFU reading reduced from 317 x 10^{11} CFU/ml to 115.7 x 10^{11} CFU/ml. On the other hand, the effect of varying the cell concentration on the degree of adhesion was compared by using cell concentration at 0.8 and 1.2 of absorbance. The results showed that at 24 hour of exposure at 0.8 and 1.2 of absorbance gave higher degree of adhesion at higher cell concentration. The adhesion of *S. aureus* and *S. cerevisiae* on the glass increased by 2.45 % and 2.36 % respectively, at higher cell concentration. Lastly, both *S. aureus* and *S. cerevisiae* gave higher percentage of adhesion at stationary phase compared to exponential phase. The percentage of adhesion at exponential state were only 22.9 % and 10.9 % for *S. aureus* and *S. cerevisiae* respectively while at stationary state the adhesion were 31.8 % and 21.3 % for respectively.

ABSTRAK

Kajian ini mendedahkan kelakuan lekatan *Staphylococcus aureus (S. aureus)* dan *Saccharomyces cerevisiae (S. cerevisiae)* pada permukaan kaca yang berkaitan dengan masa pendedahan , kepekatan sel dan umur. Oleh itu, kajian ini telah dijalankan untuk menyiasat mekanisme lekatan mikrob berdasarkan masa pendedahan, kepekatan sel dan umur budaya, supaya langkah-langkah selanjutnya boleh diambil sama ada untuk mempengaruhi atau untuk mencegah lekatan mikroorganisma. Untuk mengkaji kesan masa pendedahan mengenai proses lekatan , eksperimen telah dijalankan selama 24 jam dan persampelan diambil pada selang 4 , 8 , 12 dan 24 jam . Pada setiap kali pensampelan, slaid kaca telah diperiksa di bawah mikroskop cahaya untuk menentukan bilangan sel dilampirkan setiap persegi . Di samping itu, kuantiti dan unit membentuk koloni (CFU) juga telah dikira. Berdasarkan keputusan yang diperolehi, bacaan OD *S. cerevisiae* dan *S. aureus* menurun dengan peningkatan masa pendedahan dengan paling rendah membaca OD telah diperolehi pada 24 jam, 0.911 dan 0,827 masing-masing menunjukkan penurunan sebanyak 8.9% dan 17.3% daripada bacaan awal 1.000 . Pengurangan OD berlaku kerana bakteria telah melekat pada permukaan kaca dan pelekatan meningkat dengan masa pendedahan. Bacaan ini disokong oleh kiraan CFU , di mana bacaan awal CFU untuk *S. cerevisiae* adalah sebanyak 16 $x10^{11}$ CFU / ml dan jatuh kepada 10.3 $x10^{11}$ CFU / ml pada akhir eksperimen manakala bagi *S . aureus* yang CFU bacaan berkurangkan daripada 317 x 10^{11} CFU / ml kepada 115.7 x 10^{11} CFU / ml. Sebaliknya , kesan manipulasi kepekatan sel pada pelekatan telah dibandingkan dengan menggunakan kepekatan sel pada 0.8 dan 1.2 OD . Hasil kajian menunjukkan bahawa pada 24 jam pendedahan pada 0.8 dan 1.2 OD kedua-dua *S. aureus dan S. cerevisiae* memberikan pelekatan lebih tinggi pada kepekatan sel yang lebih tinggi dengan 2.45 % and 2.36 % masing-masing . Akhir sekali, kedua-dua *S. aureus* dan *S. cerevisiae* memberikan peratusan yang lebih tinggi lekatan pada fasa pegun berbanding dengan fasa eksponen. Peratusan lekatan pada keadaan eksponen hanya 22.9 % dan 10.9 % *S. aureus* dan *S. cerevisiae* manakala pada keadaan pegun lekatan adalah 31.8 % dan 21.3 % masing-masing.

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1 INTRODUCTION

1.1 Motivation and statement of problem

Bacteria tend to adhere to different kinds of surfaces, ranging from surfaces in the human body, and plants and clays, to plastics and metals. Once bacteria are attached to a surface, a multi-step process starts, resulting in a complex adhering microbial community called a 'biofilm' (Escher & Characklis, 1990). Biofilms can be beneficial, such as in wastewater treatment (Nicolella & Van Loosdrecht, 2000) but they may also have hazardous consequences. For instance, in water distribution systems, they may cause contamination of drinking water with pathogens such as *Legionella spp.* and *Pseudomonas aeruginosa* (Szewzyk *et al,* 2000). Biofilm formation in food-processing equipment is known to cause contamination, resulting in spoilage or disease (Kumar & Anand, 1998) while on ship hulls, biofilms are responsible for increased fuel consumption. To avoid the formation of marine biofilms, environmentally harmful antimicrobial paints have been used (Yebra *et al.,* 2004), but these have recently been banned internationally, requiring the development of non-toxic antifouling surfaces. In the medical field, the formation of biofilms on devices such as catheters and orthopaedic implants frequently constitutes a reason for device failure and removal (Gristina, 1987).

Bacterial adhesion is influenced by properties of both the bacterial and the substratum surface. Bacterial characteristics known to influence adhesion are hydrophobicity, zeta potential (Bos *et al.,* 1999) motility (Kogure *et al.,* 1998), and release of extracellular substances, such as polysaccharides (Azeredo *et al.,* 1999), proteins (Dufrene *et al,* 1996) and biosurfactants (Van Hoogmoed *et al*, 2000) .Relevant properties of the substratum surface are hydrophobicity, zeta potential (Bos *et al.,* 1999), and surface texture (Desai *et al.* , 1992; Holland *et al.,* 1998). The influence of the surface free energies of the substratum and the bacterium can be modelled using a thermodynamic approach (Bos *et al*., 1999). The extended-DLVO (Derjaguin, Landau, Verwey, Overbeek) theory accounts for Lifshitz–Van der Waals, electrostatic, and short range acid–base interaction energies between the surface and the bacterium as a function of their separation distance (Van Oss *et al.* , 1986). The mechanistic knowledge of bacterial adhesion obtained from the extended-DLVO theory provides guidelines for the development of surface coatings exhibiting minimal adhesion of bacteria.

In conclusion, it is predominated by many factors such as microbe's characteristic, surface properties and environmental conditions. This study was carried out to investigate the mechanism of microbial adhesion based on the exposure time, cell concentration and culture age, so that further measures can be taken either to influence or to prevent the adhesion of microorganism.

1.2 Objectives

The objectives of this research are as follow:

1. To study the adhesion of *S. aureus* and *S. cerevisiae* on glass surface.
2. To study the effect of physical properties on the microbial surface adhesion.

1.3 Scope of this research

The study has been divided into several scopes in order to achieve the objectives, which are:

i. Maintenance of pure culture.

ii. Microbial characterization based on the morphology, size and affinity of the microbes.

iii. Effect of physical properties on adhesion.

   a) Time

   b) Cell Concentration

   c) Culture age

1.4 Thesis Layout

The structure of this thesis was outlined as follow:

Chapter 2 details the literature review on microorganisms used, mechanism of biofilms and physical and environmental effects on adhesion.

Chapter 3 details all the general and repetitive materials and methods that were carried out throughout the study, including the preparation of culture, SEM and light microscope analysis, and the adhesion test.

Chapter 4 discusses thoroughly the effects of the physical conditions that affected the metabolic behaviour of the suspended cells towards the glass surface by varying the exposure time, the cell concentration and also the culture age.

Chapter 5 discusses the overall conclusions from the results of this study. The conclusions were derived from the results obtained during this study and the recommendations have partly highlighted some significant findings that can contribute to future improvement.

2 LITERATURE REVIEW

2.1 Staphylococcus aureus

Staphylococci family are Gram-positive bacteria with diameter of 0.5 – 1.5 µm as seen in Figure 2.1 and was characterized by individual *cocci* which divide in more than one plane to form grape-like cluster. Until now, there are 32 species and 8 subspecies in the genus of *Staphylococcus*. However, *S.aureus* and *S.epedermis* are the two most characterized and studied strains. They are non-motile, non- spore forming anaerobes that grow either by aerobic respiration or fermentation. *S.aureus* is more virulent compared to *S.epedermis* despite their phylogenic similarities. The cell wall of *S.aureus* is a tough protective coat which is relatively amorphous in appearance, about 20-40 nm thick. The growth and survival of bacteria is dependent on the cell ability to adapt to environmental changes. *S.aureus* has evolved many mechanisms to overcome these changes. In fact, *S.aureus* has been found to be a common cause of various infections on biomaterial surfaces. Biomaterial surfaces usually have a negative charge and initially repel the negatively charged bacteria. However, at a distance of around 15 nm, van der Waals and hydrophobic forces are exerted and repulsion is overcome (Harris, 2002).

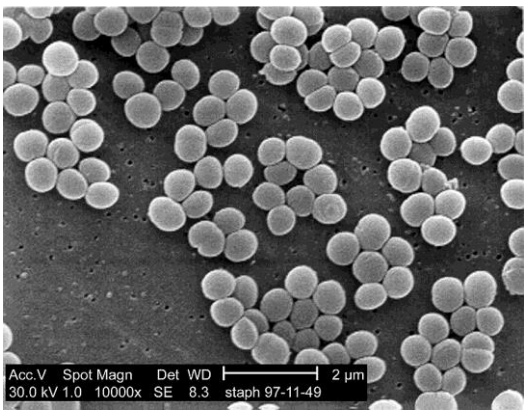

Figure 2 - 1 Scanning Electron Microscope (SEM)'s image of *Staphylococcus aureus* (Carr, 2007)

2.2 Saccharomyces cerevisiae

Yeast or scientifically *Saccharomyces cerevisiae* is widely used in production of ethanol. *S.cerevisiae* is a unicellular microorganism which belongs to the fungi group. Typically *S.cerevisiae* is either spherical or oval in shape, with clear internal cell structures. Most of them are unicellular and their size can vary from 3-5 µm in diameter, although some can reach 7 µm. Normally *S.cerevisiae* grows by budding (the common *S.cerevisiae*), but very rarely can multiply by binary fission. The surface is normally negatively charged and easily immobilised on solid surfaces. Yeast in general is hydrophobic therefore it prefers hydrophobic surfaces, and attaches weakly to hydrophilic surfaces such as on glass. However, stronger adhesion of the yeast-surfaces can be achieved by introducing electrostatic interaction between the yeast and the solid surfaces (Norton & D'Amore, 1994)

S.cerevisiae is well built with vacuole, mytochondria, cytoplasm and nucleus, and the outer membrane is made of glucan and polysaccharides. The surface is covered with protein and peptidoglycan, which gives it hydrophobic properties. The surface protein also results in a negatively charged surface in *S.cerevisiae*, but can vary significantly depending on the environment (medium types), pH and ionic strength. The surface charge of the yeast is also dependant on the age, and normally loses its negativity as it enters the stationary phases. The dead cells of yeast are generally neutral in charge (Zain, 2012).

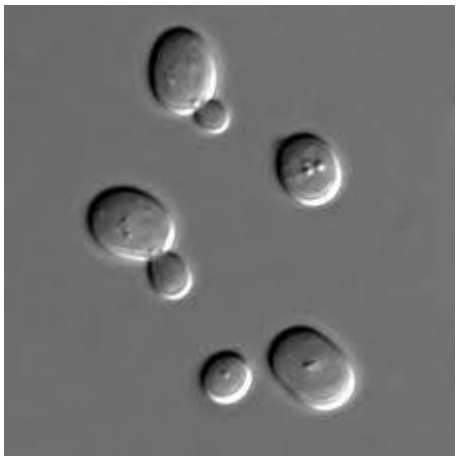

Figure 2 - 2 Differential interference contrast (DIC) microscopy image of *Saccharomyces cerevisiae* (Masur, 2010)

2.3 Microbial Activity

Compared to algae and fungi, bacteria regarded as more versatile because they are not limited by the need for light or a consumable substrate. Besides, due to differences in properties of cell surface, there is considerable variation among bacteria (Consterton *et al*, 1978). Bacteria attached to a surface appear to be metabolically different from their planktonic or 'free swimming' counterparts (Fletcher, 1992). Meanwhile, fungi require a fixed organic source of carbon. Their rigid cell walls limit them to being saprophytic on organic substrates or as parasites on animal. Moreover, fungi may be found on any solid that provides an organic substrate, provided that the local conditions are satisfactory (Bott, 2006).

Microbial cells are surrounded by a cell wall, which retain the cell contents and is the primary barrier between the cell surface and the environment in which it exist. The quality of cell wall in terms of selective permeability, maintains the necessary levels of nutrients, trace elements and cell internal pH. The cell membrane is the site of transfer process: water is able to pass through this membrane, in or out the cell depending on the trust of the osmotic pressure. The chemistry of the cell wall affects its properties in terms of surface electric charge and the availability of binding ions (Bott, 2006).

The rigidity of cell wall allows the development of structure that may be beneficial in the maintenance of a coherent biofilm. Microbes produce extracellular materials such as slimes of polysaccharides and mucilage, which may help to maintain attachment on solid surfaces, provide sources of nutrients in case of nutrients availability decline or enhance protection of the cells. Clearly, the availability of nutrient determines the development of a biofilm (Bott, 2006).

2.4 Biofilms

In nature, bacterial cells are most frequently found in close association with surfaces and interfaces, in the form of multicellular aggregates embedded in an extracellular matrix generally referred to as biofilms (Donlan, 2002). Biofilms are usually heterogeneous; in that they contain more than one type of bacterial species, but they can be homogeneous in cases such as infections and medical implants (O'toole *et al.*, 2000). Microbial biofilms pose a challenge in clinical and industrial setting especially in food

processing environments where they act as a potential source of microbial contamination of foods that may lead to spoilage and transmission of foodborne pathogens (Houdt & Michiels, 2010); (Adetunji & Isola, 2011). They can also compromise the cleanliness of food contact surfaces and environmental surfaces by spreading detached individual microorganisms into the surrounding environment (Milanov *et al.*, 2009). Environmental conditions in food production areas including the presence of moisture, nutrients, and inoculum of microorganisms from the raw materials might favour the formation of biofilm. Furthermore, when food processing equipment are not easily cleaned due to its design and food particles not completely removed, the particles aid in the formation of biofilms by providing a coat that not only provides the biofilm with nutrients but also a surface to which it can easily stick on (Kamila & Katarzyna, 2011). Once biofilms have formed on food processing surfaces, they are hard to eliminate often resulting in persistence and endemic population. Biofilms offer their member cells several benefits, including channelling nutrients to the cells and protecting them against harsh environments.

In particular, it has been noted that cells within biofilms are more resistance to antibiotics, disinfectants, and to host immune system clearance than their planktonic counterparts (Houdt & Michiels, 2010); (Morikawa, 2006). Several mechanisms account for this increased antibiotic resistance, including the physical barrier formed by exopolymeric substances, a proportion of dormant bacteria that are inert toward antibiotics, and resistance genes that are uniquely expressed in biofilms (Kavanaugh & Ribbeck, 2012). Outbreaks of pathogens associated with biofilms have been related to the presence of species *of Listeria, Yersinia, Campylobacter, Salmonella, Staphylococcus,* and *Escherichia coli* O157:H7. These bacteria are of special significance in ready-to-eat and minimally processed food products, where microbiological control is not conducted in the terminal processing step (Kamila & Katarzyna, 2011).

2.5 Formation of Biofilms

Adhesion of bacteria will form a colony (biofilm) consisting prokaryotes cells, surrounded by matrix of biomolecules secreted by the cells. In the creation of biofilm, even though the structure and function is different for different bacteria, this same four step always been followed. Firstly, small molecules, initially water and salt ions will absorb to the surface. Hence, the substrate surface will be covered with a single layer of small molecules or proteins that present in the medium. Conditioning film consists of mixture of water, ions and proteins are always present before the first microorganisms arrive at the surface. The second step is characterized by the initially reversible adsorption of microorganisms to the conditioning film. Then will be the arrival of microbes either by Brownian motion, gravitation, diffusion or intrinsic motility. They may adhere to each other forming microbial aggregates before absorbing to the conditioning film. Since microbes adhere to conditioning film and not the surface itself, the strength depends on the structure of the conditioning film. The initially reversible adsorption becomes irreversible, mainly through the secretion of exopolymeric substances by the adsorbed microorganisms in step three. These substances will incorporate in the conditioning film and strengthen its cohesiveness. Finally, the number of microorganisms in the biofilm accumulates mainly through in situ cell growth (T. Boland, 2000).

2.6 Effect of Physical Properties

2.6.1 Effect of Temperature

Besides, each microorganism has their own optimum temperature where when sufficient nutrients are available, the growth is maximum. The optimum temperature is different to different species on account of various metabolic characteristics ranging from 20–50 °C with mainly between 35-40 °C (Bott, 2006). Nutrient metabolism is directly related and dependent on presence and reaction rates of enzymes. Thus, temperature and reaction rates of enzymes do correlates. At optimum temperature, the bacteria give healthy population growth while temperature far from optimum reduces the growth efficiency due to reduction in reaction rate of enzymes (Trevor *et al.*, 2008).

On the other hand, the physical properties of the compounds within and surrounding the cells were also affected by the environmental temperature. Findings showed that a decrease in temperature reduced the adhesive properties of a marine Pseudomonad (Fletcher , 1977). He believed that the effect was due to a decrease in the bacterial surface polymer at lower temperatures as well as effects such as reduced surface area. However, Herald & Zottola, 1988 observed that the presence of bacterial surface appendages was dependent on temperature. At 35 °C cells were shown to have a single flagellum whilst at 21 °C they had two to three flagella and at 10 °C, cells exhibited several flagella.

2.6.2 Effect of pH

pH changes can significantly affect bacterial growth and frequently exploited in the production of detergents and disinfectants used to kill bacteria. Bacteria possess membrane-bound proton pumps which extrude protons from the cytoplasm to generate a transmembrane electrochemical gradient which called the proton motor force (Rowland, 2003). The passive influx of protons in response to the proton motive force can be a problem for cells attempting to regulate their cytoplasmic pH (Booth, 1985). Large variations in external pH can overwhelm such mechanisms and have a biocidal effect on the microorganisms. Bacteria respond to changes in internal and external pH by adjusting the activity and synthesis of proteins associated with many different cellular processes (Olsen, 1993). Research has shown that a gradual increase in acidity increases the chances of cell survival in comparison to a sudden increase by rapid addition of HCl (Li, 2001). This suggests that bacteria contain mechanisms in place which allow the bacterial population to adapt to small environmental changes in pH. However, there are cellular processes which do not adapt to pH fluctuations so easily. One such process is the excretion of exopolymeric substances (polysaccharides). Optimum pH for polysaccharide production depends on the individual species, but it is around pH 7 for most bacteria (Oliveira,*et al.*, 1994).

2.6.3 Effect of Bulk Water Velocity

Basically, the effect of bulk water velocity is twofold. Velocities are proportional to turbulence in the bulk flow and inversely proportional to thickness of the boundary layer adjacent to the biofilm residing on the solid surface. As the velocities increases, the availability of nutrients at certain concentration, to the biofilm increases because of the lower resistance to mass transfer of nutrients to the biofilm. However, as the velocity increases, the attendant shear forces acting on the biofilm also increases. This explained the reduction of biofilm accumulation. Thus, it was proven that the increase growth rate was because of greater nutrient availability and the removal of biofilm by the increased velocity (Grant & Bott, 2005).

2.7 Effect of Surface Characteristics

2.7.1 Effect of Surface Hydrophobicity

During the adhesion process, bacteria firmly adhere to the biomaterial surface through physicochemical interactions. These comprise cell surface hydrophobicity and charge as well as the hydrophobicity, charge, roughness, and chemical composition of the biomaterial surface itself. Surface hydrophobicity, in particular, has been described as one of the most important properties involved in the adhesion phenomenon. According to van Oss and Giese, in biological systems, hydrophobic interactions are normally the strongest of the long-range non-covalent interactions and can be defined as the attraction among apolar, or slightly polar, cells or other molecules themselves, when immersed in an aqueous solution (Sousa, *et al.*, 2009).

In the course of the short-term phase of microbial adhesion surface properties of bacteria and substrata (-potential and hydrophobicity) together with the composition of the liquid medium have been recognized as determining whether adhesion will be effective or not. Primarily there is much evidence on the crucial role of degree of wettability of substrata. In the same way, microorganisms can be separated roughly into three categories: (a) hydrophobic (having water contact angle $ø_w$, > 90°); (b) moderately hydrophobic ($ø_w$, = 40-90°) and (c) hydrophilic ($ø_w$, < 400°). The following general features were identified: (1) Hydrophobic bacteria adhere to hydrophobic surfaces irreversibly in the so-called primary minimum even at strong electrostatic repulsion. (2)

Moderately hydrophobic bacteria adhere reversibly in the so-called secondary minimum and, when the surfaces are hydrophobic, again irrespective of the electrostatic interaction. (3) Hydrophilic bacteria adhere to both hydrophilic as well as hydrophobic surfaces in the secondary minimum. Adhesion of these bacteria (as well as of any bacteria to hydrophilic surfaces) is relatively weak and reversible and mostly requires electrostatic attraction (Skvarla, 1993).

Besides, Makin & Beveridge, 1996 were able to show that cell surface hydrophobicity was the primary mediator of adhesion of *Pseudomonas aeruginosa* strains to hydrophobic surfaces, whereas for hydrophilic cells, surface charge played a major role. Like most microorganisms, the species used in our study were all negatively charged. It was therefore expected that they would preferentially adhere to surfaces with a positive charge. Though the adhesion of *S. wolfei* cells was indeed significantly increased on Fe31-coated surfaces, adhesion of the other species was less or not at all affected. Based on cell surface hydrophobicity measurements (by both HIC and BATH testing) we expected increased adhesion of *Desulfovibrio* sp. strain G11 and *D. tiedjei* cells on hydrophobic surfaces. However, siliconecoated surfaces inhibited adhesion of all the strains, including the relatively hydrophobic *D. tiedjei* cells. Such discrepancies are difficult to explain, but gross measurement of surface properties such as charge and hydrophobicity does not always consistently correlate with attachment or transport through porous media (Kjelleberg & Hermansson, 1984)

2.7.2 Effect of Surface Roughness

Biomaterial surface roughness is another property relevant for the bacterial adhesion process, with the irregularities of the polymeric surfaces normally promoting bacterial adhesion and biofilm accumulation. This is due to the increased surface area and depressions that provide more favourable and additional sites for colonization, as such crevices protect bacterial cells from the shear forces. However, the accumulation of bacteria in such locations depends largely on their size, cell dimension, and division mode. In fact, according to some authors, a linear relation of bacterial adhesion with surface roughness is not always verified. A small increase in roughness can lead to a significant increase in bacterial adhesion, while a larger increase in roughness can have no significant effect on cellular attachment (Sousa, *et al.*, 2009).

2.7.3 Effect of Surface Topography

The results of Hsu's work clearly show that substrate surface topography at the micro- and nanoscale affects bacterial attachment. Cells seem to try to maximize contact area with the surfaces, presumably to achieve a stronger and more stable attachment, which results in a specific alignment of the cells depending on the arrangement of the topographical details. Moreover, surface topography appears to induce the expression of different types of appendages that might mediate attachment. Better understanding of the way in which bacterial cells attach to surfaces with controlled topography in the micro- and nanoscale will allow the design and fabrication of materials able to effectively control bacterial adhesion, with a large number of potential biomedical and industrial applications (Hsu, *et al.*, 2013).

2.8 Glass

Glasses are a type of ceramic materials presenting vitreous structures derived from silica, which are formed by the bonding of the Si–O tetrahedrons or other ionic groups, in order to produce a non-crystalline but solid lattice structure. The basic building block of silicate structures is the SiO_4 tetrahedron, exhibiting linkages with a strong covalent character (White, 2003). In addition to oxides of silicone, glass can be obtained by other kind of oxides such as B_2O_3 or Al_2O_3, both of them known as glass precursors. There are also other oxides which can be added to the glasses, called modifiers. Among these elements, alkali and alkaline earth oxides are commonly used to reduce its viscosity and thus enhance process ability properties and ease of shaping of the final material. It is necessary to point that the unit structure of silicate tetrahedral is maintained in the crystal, regardless the oxides that could be added in order to modify its properties.

A typical borosilicate glass microscope slides will be used in this research as these slides are widely available. Borosilicate glass is a type of glass with the main glass-forming constituents' silica and boron oxide. Borosilicate glasses are known for having very low coefficients of thermal expansion (~3×10^{-6} /°C at 20°C), making them resistant to thermal shock, more so than any other common glass. Such glass is less subject to thermal stress and is commonly used for the construction of reagent bottles.

Borosilicate glass is sold under such trade names as Suprax, Kimax, Pyrex, Endural, Schott, or Refmex.

3 MATERIALS AND METHODS

3.1 Chemicals

Glucose, bactopeptone, yeast extract, nutrient agar, sulphuric acid, NaoH, $K_2HPO_,$ $KH_2PO_{4,}$ KCl, $MgSO_4$, NaCl and glutaraldehyde were obtained from FKKSA Laboratory, UMP.

3.2 Preservation of Culture

Saccharomyces cerevisiae and *Staphylococcus aureus* were obtained from the Centre Laboratory of Universiti Malaysia Pahang. For long term preservation, the culture was kept in agar plates and agar slants, in a freezer at 4 ^{o}C. For use in subsequent microbial work, the yeast and *S. aureus* stock was stored in the chiller at 4-6 ^{0}C, transferred to an agar plate and incubated for 24 h at 30 ^{o}C.

3.3 Media Preparation

3.3.1 Preparation of Nutrient Broth

8 g of nutrient broth powder which made up of 20 g/L glucose, 20 g/L bactopeptone and 10 g/L yeast extract, and adjusted to pH 5.5 using 0.1 M sulphuric acid and 0.1 M NaOH solution was weighted. The powder then added to 1L of distilled or deionized water in a 1 L Schott bottle. The powder was dissolved completely in the water. It is finally autoclaved at 121 °C for 20 minutes.

3.3.2 Preparation of Nutrient Agar

20 g of nutrient agar powder containing 20 g/L glucose, 1.5% (w/v) agar powder, 20 g/L bactopeptone and 10 g/L yeast extract, and adjusted to pH 5.5 using 0.1 M sulphuric acid and 0.1 M NaOH solution was weighed out. 1 L of distilled or deionized water in a 1 L Schott bottle was added. It is finally autoclaved at 121 °C for 20 minutes and cooled to 50 °C before pouring into the petri dish.

3.3.3 Preparation of Agar Plates

15-20 mL of a warm sterile nutrient agar was poured per petri plate. The nutrient agar then allowed to solidify at room temperature in sterile environment and kept in 4 °C until further use.

3.3.4 Preparation of Phosphate Buffer Saline (PBS) Solution

PBS solution was prepared according to the specific composition to get 7.0 of desired pH. The solution was autoclaved at 121 ^{o}C for 20 minutes prior to use.

Table 3 - 1 Composition of PBS solution

Material	**Amount**
1M K_2HPO_4	0.802 mL
1M KH_2PO_4	0.198 mL
5M KCl	1.0 mL
0.1M $MgSO_4$	1.0 mL
Distilled water	97.0 mL
NaCl	0.85

3.4 Culture Preparation

3.4.1 Germination of Stock Culture and Inoculum

A loopful of refrigerated stock culture was transferred onto a petri dish containing medium agar and incubated at 30 ^{o}C. After 24 hours of incubation, a colony of germinated cells was transferred to a 250 mL shake flask containing 30 mL of growth medium (without agar), then placed in an orbital shaker at 180 rpm, for 16 hours. The cells were then centrifuged at 5000 rpm for 5 minutes, washed once with 0.85 % (w/v) NaCl, and re-centrifuged for 3 minutes (Jamai *et al*., 2001). The supernatant was discarded and the pellet was suspended in saline solution by vortexing. The total cell

concentration was adjusted to an absorbance of approximately ~1.0 at 600 nm using a UV-Vis spectrophotometer.

3.4.2 Preparation of Seed Culture

About a plate of microbes samples are taken transferred into 100 mL nutrient broth. The broth then allowed to be incubated for 16 h, 18 h and 66 h depending on the type of experiments.

3.5 The characterization of *S. aureus* and *S. cerevisiae*

3.5.1 Viewing Cell using Light Microscope

Samples at exponential and stationary state were smeared on the glass slides and images were viewed through light microscope in FKKSA laboratory, UMP. The images were captured and characteristics were examined using Dino-Eye Piece camera attached to a computer.

3.5.2 Viewing Cell Using Scanning Electron Microscope

The same samples used in light microscopy were also sent to Central Laboratory; UMP to be examined using SEM analysis which later used for comparison of both methods.

3.5.3 Cell Surface Hydrophobicity/Microbial Adhesion to Solvents (CSH/MATS)

The characteristics of the cells have been reported to be influenced by the cells' age and density/concentration. Cell suspensions were prepared from the 16 h and 66 h cultures to study the cells' surface characteristics during the exponential and stationary phase. Four types of solvents were used to determine the electron donor/acceptor properties of the cells. 4 ml solvent was added to the 4 ml of cell suspension with the optical density fixed at 1.000, read at 600 nm with a UV spectrophotometer. The mixture was then mixed and vortexed for 60 seconds and allowed to separate at room temperature for 15 minutes. Finally the optical density of the cell suspension (aqueous phase) was measured against a blank (distilled water). All measurements were carried out in triplicate and the results presented were the average values.